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Nutrient availability affects floral scent much less than other floral and vegetative traits in *Lithophragma bolanderi*

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• **Background and Aims** Many plant–pollinator interactions are mediated by floral scents that can vary among species, among populations within species and even among individuals within populations. This variation could be innate and unaffected by the environment, but, because many floral volatiles have amino-acid precursors, scent variation also could be affected by differences in nutrient availability among environments. In plants that have coevolved with specific pollinators, natural selection is likely to favour low phenotypic plasticity in floral scent even under different conditions of nutrient availability if particular scents or scent combinations are important for attracting local pollinators.
• **Methods** Clonal pairs of multiple seed-families of two *Lithophragma bolanderi* (Saxifragaceae) populations were subjected to a high and a low nutrient treatment. These plants are pollinated primarily by host-specific *Greya* moths. It was evaluated how nutrient treatment affected variation in floral scent relative to other vegetative and reproductive traits.
• **Key Results** Floral scent strength (the per-flower emission rate) and composition were unaffected by nutrient treatment, but low-nutrient plants produced fewer and lighter leaves, fewer scapes and fewer flowers than high-nutrient plants. The results held in both populations, which differed greatly in the number and composition of floral scents produced.
• **Conclusions** The results reveal a strong genetic component both to scent composition and emission level, and partly contrasts with the only previous study that has assessed the susceptibility of floral volatile signals to variation in the abundance of nutrients. These results, and the tight coevolutionary relationship between *Lithophragma* plants and their specialized *Greya* moth pollinators, indicate that reproductive traits important to coevolving interactions, such as the floral scent of *L. bolanderi*, may be locally specialized and more canalized than other traits important for plant fitness.

Key words: *Lithophragma bolanderi* (Saxifragaceae), floral scent, canalization, adaptation, coevolution, environmental effects, floral volatiles, nutrients, phenotypic plasticity, local specialization, 1,4-dimethoxybenzene

INTRODUCTION

Much of the spectacular trait and species diversity of flowering plants can be attributed to the evolution of flowers and interactions between plants and pollinators (Kay *et al.*, 2006; Kay and Sargent, 2009; van der Niet and Johnson, 2012; Armbruster, 2014). Floral trait variation is often conserved at the species level, and is more canalized than variation in vegetative traits in response to environmental fluctuations (Berg, 1960; Armbruster *et al.*, 1999; Hansen *et al.*, 2007; Pélabon *et al.*, 2011, 2013). Such canalization and reduced variation in floral traits implies that the plant–pollinator interaction often imposes strong selection for certain floral phenotypes (Cresswell, 1998; Rosas-Guerrero *et al.*, 2011; Pélabon *et al.*, 2013). Indeed, floral phenotypes are commonly reported as subject to pollinator-mediated selection (Galen, 1989; Campbell *et al.*, 1997; Alexandersson and Johnson, 2002; Sandring and Ågren, 2009; Sletvold *et al.*, 2010), which, when acting in different directions in different populations, could lead to speciation (Campbell, 2003; Anderson *et al.*, 2009; Kay and Sargent, 2009).

Most of our understanding of floral trait variation comes from studies of visual or morphological traits (e.g. colour, shape), and systematic studies of chemical trait variation have only recently become a focus of study (e.g. Dötterl *et al.*, 2005; Raguso, 2008; Schiestl and Johnson, 2013; Parachnowitsch, 2014; Parachnowitsch and Manson, 2015). Often, however, studies of floral scent variation are performed under field settings, and thus focus on phenotypic variation (Parachnowitsch, 2014). Hence, it can be difficult to distinguish variation due to differences in the genetic make-up of the target individuals, populations or species from environmentally induced variation such as shading, temperature or access to nutrients. The few studies that have experimentally evaluated plasticity in floral scent have typically focused on the impact of the daily (night/day) rhythm and/or temperature variation (Matile and Altenburger, 1988; Raguso *et al.*, 2003; Hoballah *et al.*, 2005; Majetic *et al.*, 2009; Dötterl *et al.*, 2012; Hu *et al.*, 2013; Friberg *et al.*, 2014; Farré-Armengol *et al.*, 2014). A few studies have compared scent variation between natural sites and greenhouse common gardens (Majetic *et al.*, 2009; Friberg *et al.*, 2014)

and one recent study has found varying effects of drought on the floral scent of different plant species (Burkle and Runyon, 2016). Also, only a single, very recent, study (Majetic *et al.*, in press) has investigated a potential impact of nutrient variation on floral scent production and composition. This paucity of studies is quite surprising, because nutrient levels are known to affect other aspects of plant chemistry (Bryant *et al.*, 1987; Mutikainen *et al.*, 2000; Ballhorn *et al.*, 2011; Miehe-Steier *et al.*, 2015).

The access to nutrients can vary among populations and among microhabitats within populations. Many floral volatiles are produced in synthetic pathways with nitrogen-containing amino acid precursors (Weaver and Herrmann, 1997; Pichersky, 2006), and nitrogen is a common limiting factor for terrestrial plants (Chapin *et al.*, 1987; Vitousek and Howarth, 1991; Gruber and Galloway, 2008). Therefore, variation in nutrient environment could affect both the amount of volatiles released and the composition of the scent signal, if certain volatile compounds are costlier to produce than others. Indeed, such effects have recently been reported from *Petunia hybrida* (Majetic *et al.*, in press), where one compound, eugenol, which is attractive to their bee pollinators, is significantly affected by nitrogen availability. The emission of most floral compounds investigated in the Petunias was, however, not affected by the nitrogen treatment (Majetic *et al.*, in press), suggesting that particular floral scent compounds, or combinations of compounds could be quite canalized and less plastic in response to nutrient environment than many other reproductive or vegetative traits. Such canalized variation is reported for many morphological floral traits (Mal and Lovett-Doust, 2005; Brock and Weinig, 2007; Burkle and Irwin, 2009; Rosas-Guerrero *et al.*, 2011), indicating that the ability to present particular floral shapes could be tightly linked to fitness. Likewise, if certain floral scent combinations are largely unaffected by nitrogen treatment, that would suggest that a particular combination of compounds is important for attracting the local suite of pollinators and that divergence in scent composition among populations is probably shaped by local specialization.

In some cases, such as in pollinating floral parasites involved in nursery pollination systems, local canalization for floral scent may be particularly strong because plants attract single highly specialized pollinator species. At the extreme, some species of figs (e.g. Chen *et al.*, 2009) have evolved particular compounds that attract their highly specialized and coevolved fig wasp pollinators. A similar 'private channel' of communication is suggested but not yet determined between Yuccas (Asparagaceae) and Yucca moths (Prodoxidae), and the yucca scent bouquet varies little among the populations and species that have been studied (Svensson *et al.*, 2005, 2006, 2011). Similarly, *Lithophragma* (Saxifragaceae) plants are pollinated by other specialized prodoxid moths (*Greya* moths), but they differ from yuccas in producing a diverse array of floral volatile compounds within populations and strong scent divergence among species and populations (Friberg *et al.*, 2013, 2014). The specificity of the *Lithophragma–Greya* interaction is known to be at least partially mediated by the floral scent, because moths are particularly attracted to the floral scent of the local *Lithophragma* species (Friberg *et al.*, 2014, 2016). We can therefore predict that despite the great among-species and among-population diversity of compounds emitted by *Lithophragma*, these plants should be canalized locally in response to environmental variation in nutrient availability.

We experimentally tested the impact of nutrients on floral scent variation in two populations of woodland stars (*Lithophragma bolanderi*). We used a paired design, exposing different individuals of the same clones to a low- and a high-nutrient treatment and investigated how population affiliation and nutrient treatment affect quantitative and qualitative variation in floral scent as compared with a set of vegetative and reproductive traits. Our results demonstrate that whereas the number of leaves, scapes and flowers, as well as the colour of the leaves, were all significantly affected by nutrient levels, floral scent was much more canalized and similar both in scent composition and in emission rate across treatments.

MATERIALS AND METHODS

Study system

Lithophragma bolanderi is distributed across the Sierra Nevada, CA, USA, and is pollinated by the prodoxid moth *Greya politella*. Adults mate on and take nectar from the flowers, and females oviposit through the corolla into the ovary, during which pollen from other flowers adhering to the female abdomen pollinates the flower (Thompson and Pellmyr, 1992; Thompson and Cunningham, 2002; Thompson *et al.*, 2010, 2013). In some populations plants are visited also by generalized pollinators, and in some rare cases bombyliid flies or solitary bees can be sufficiently common to swamp the mutualism between *Lithophragma* and *Greya* (Thompson and Cunningham, 2002; Thompson and Fernandez, 2006; Cuautle and Thompson, 2010). Several *Lithophragma* species show ample within-species divergence in the floral scent signal. This divergence is particularly evident in *L. bolanderi*; in some natural populations the scent bouquet of most (or all) individuals is dominated by the benzenoid ether 1,4-dimethoxybenzene (1,4-DMB), whereas most or all plant individuals of other populations do not emit this compound (M. Friberg *et al.*, unpubl. data). Populations of *L. bolanderi* also emit a variety of other floral volatiles, raising the question of whether the observed variation reflects environmentally caused differences in floral scent or genetic differences among populations. Here, we assess how nutrient levels affect floral scent variation in two populations of *L. bolanderi*: one in which field samples were dominated by 1,4-DMB (Woody, CA: 35°43·176′N, 118°47·907′W; M. Friberg *et al.*, unpubl. data), and one in which most individuals lacked this compound (Marble Falls, Sequoia National Park: 36°31·198′N, 118°48·024′W; Friberg *et al.*, 2014).

Plant growth

Seeds from 20 maternal families, ten from each population, were collected in the field and planted in the greenhouse to produce root bulbils (Table 1). Each bulbil is a vegetative reproductive root mass that *Lithophragma* plants produce at the end of the spring growing season and that then produces clonal leaves and scapes the next spring. Three bulbils derived from different seed individuals were planted from each seed family and cut with a razor blade into 4–6 clonal pieces. These

TABLE 1. *The planting scheme and sample sizes in the experiment*

Planted				Sample sizes (sample size/clonal pairs/seed families)					
Population	Seed families	Seed family individuals	Clones	Leaves	Scapes	Scape height	Flowers	Colour	Floral scent
Marble Falls	10	3 per seed family	2–6 per seed family individual	32/16/10	36/18/10	32/16/8	32/16/10	34/17/10	36/18/10
Woody	10	3 per seed family	2–6 per seed family individual	16/8/6	22/11/6	22/11/6	18/9/6	18/9/6	18/9/6

Bulbils from three seed individuals (these are the bulbil 'offspring' of different seedlings from a field-collected seed family) of ten different seed families/population were planted. Each bulbil was separated into two to six similarly sized pieces (depending on original size). Half of these bulbil pieces for each seed family individual were planted into the high- and the low-nutrient treatment, respectively. The sample sizes on the right-hand side of the table report the total sample sizes, i.e. the number of cases for when a clonal pair (from the same seed family individual) was flowering in both the high- and the low-nutrient treatment and data were available for number of leaves, number of scapes, scape height, number of flowers, colour of the leaves and floral scent. Hence, some of these clonal pairs came from different seed individuals from the same seed family and were thus at least half-sibs. For more information on flowering rates and planting scheme, please see Supporting Data, Table S1.

pieces were planted in individual 2·5-inch pots (Percival Model I36LLVL) in Pro-Mix 'BX' (Mycorise Pro) potting soil. Two plants (i.e. two individuals growing in different pots) (1) could belong to the same or different populations, (2) and within populations could belong to the same or different seed families. Furthermore, (3) in some cases, two plants could belong to the same seed family but descend from different seed individuals (i.e. being half- or full sibs), and finally (4) two plants could descend from the same seed individual and thus be genetic clones.

Half of the pots with clones of each seed family individual were assigned to a high-nutrient treatment, and the other half was assigned to a low-nutrient treatment (Table 1). All plants were watered on Mondays, Wednesdays and Fridays, and fertilized once per week with Dyna-Gro liquid 7-9-5 fertilizer containing 7 % nitrogen (NH_4 and NO_3), 9 % phosphorous (P_2O_5) and 5 % potassium (K_2O), beginning one week after planting and ending when the plants stopped producing photosynthetic pigments. The high-nutrient group was fertilized with 15 mL per 3 L water, and the low-nutrient group with 2·5 mL per 3 L water. Light, temperature and humidity were controlled at each growth stage. Plants were initially grown in an incubator (Percival, Boone, IA, USA), with 15 °C at day, and 10 °C at night (fluorescent lights set for a 14:10-h light–dark photoperiod) for 5 weeks, then moved to a growth chamber for 2·5 weeks (Conviron E-15, Pembina, ND, USA, 15 °C day, 10 °C night, fluorescent and incandescent lights set for a 14:10-h light–dark photoperiod, 70 % relative humidy), and finally transferred to semi-humid conditions in a greenhouse equipped with a swamp cooler (~20 °C) and overhead lamps until senescence.

Data collection

Reproductive effort for each plant was measured as the number of scapes, the number of flowers and the height of scapes. The total number of leaves was recorded for each plant used for scent collection; the average foliage colour was recorded using an Ocean Optics USB2000 spectrophotometer with a PX-2 pulsed xenon lamp to measure the reflectance of five random leaves from each plant, and analysed using the OOIBase software (Ocean Optics, Dunedin, FL, USA). The spectrum from each leaf was taken from the middle region of the adaxial surface of the leaf, and the spectral measurement area was 2 mm^2. We followed the protocol described by Friberg *et al.* (2014) to calculate mean reflectance for each sample across colour spectra (ultraviolet: 300–380 nm wavelengths; violet: 381–450 nm; blue: 451–475; cyan: 476–495 nm; green 496–570 nm; orange: 571–590 nm; yellow: 591–620 nm; and red: 621–700 nm), using the Excel-based programs BinR1.7 and ColoR 1.7 (Montgomerie, 2006). The reflectance values from these five leaves were then averaged for each colour spectrum for each plant.

Floral volatiles were collected using dynamic headspace followed by hexane elution, using a sample of 5–10 flowers per plant, following the protocol described by Friberg *et al.* (2013). Scent was collected for 2 h, starting between 0930 and 1300 h in a designated room held at room temperature (~20 °C), with fluorescent overhead lighting (see Friberg *et al.*, 2013). From each plant, flowers attached to the scapes, were sealed in an 8×14-cm Reynolds® oven bag with a small hole in the top and a scent trap containing a Tenax GR® (10 mg) filter. The trap was connected by vinyl tubing to a Cole-Parmer (Vernon Hills, IL, USA) 65-mm direct-reading flow meter, which was then connected to a laboratory vacuum nozzle pulling air through the bag at a steady flow of 200 mL air per minute. Floral scent was collected in bouts of up to ten samples between 16 April and 5 June 2015. Plants were chosen based on the number of flowers available at the time of scent collection. When possible, we tried to include samples of both populations and nutrient treatments in each bout to avoid any bout effects. For every collection bout, a negative control of ambient air was collected using the same equipment and techniques as for the regular samples. Then, scent traps were eluted with 300 µL of GC/MS quality hexane, and the samples were concentrated to 50 µL under a constant flow of nitrogen gas (N_2). An internal standard of 5 µL of a 0·03 % toluene solution in hexane was added to each sample after concentration.

Scent samples were analysed using gas chromatography/mass spectrometry (GC/MS) on a Hewlett-Packard (HP) 5890 chromatograph connected to an HP 5971 spectrometer (electronic ionization). The gas chromatograph was equipped with a polar EC WAX column (30 m, 0·25 mm × 0·25 µm film thickness; Grace, Deerfield, IL, USA). Helium was used as the carrier gas at a constant velocity of 1 mL min^{-1}. Samples were analysed starting with a 3-min holding period (60 °C). Then the GC temperature was increased by 10°C min^{-1} for 20 min until it reached a maximum of 260 °C, at which it stayed for 7 min.

Chromatograms were manually integrated using the MS manufacturer's software (G1034 Version C.02.00; Hewlett-Packard 1989–1993). Floral volatiles were identified by the combined use of MS library suggestions (NIST/Wiley), comparison with literature retention indices and co-chromatography with synthetic standards (Supplementary Data, Table S1). The floral scent data were prepared for analysis by estimating the standardized emission rate [(ng scent per flower) h^{-1}; see e.g. Svensson *et al.*, 2005; Friberg *et al.*, 2013)], for all compounds in all samples. The standardized total scent emission (sum of all floral volatiles) was calculated for each sample. A handful of the floral scent samples (three of 54) included the common aliphatic wounding compounds 3-hexen-1-ol and 3 hexen-1-ol acetate. These compounds were not included in the statistical analysis.

Statistical analysis

All analyses were performed in the statistical software R (version 3.3.0). First, we tested whether the different nutrient treatments affected sprouting and flowering, using the R-package lme4, with population and nutrient treatment as categorical predictors and logit as the link function. In total, 80 % (202/252) of the planted bulbils produced leaves, and there was no significant effect of nutrient treatment or population on sprouting frequency (mixed generalized linear model: population $\chi^2_1 = 0{\cdot}15$, $P = 0{\cdot}70$; nutrient treatment $\chi^2_1 = 0{\cdot}58$, $P = 0{\cdot}44$, population × nutrient treatment $\chi^2_1 = 0{\cdot}65$, $P = 0{\cdot}42$). Sixty-two per cent ($n = 126$) of the sprouting plants produced flowers. A higher percentage of plants from Marble Falls flowered than plants from Woody, and a higher percentage of high-nutrient plants flowered in both populations (mixed generalized linear model: population $\chi^2_1 = 10{\cdot}9$, $P < 0{\cdot}001$; nutrient treatment $\chi^2_1 = 7{\cdot}07$, $P = 0{\cdot}0078$, population × nutrient treatment $\chi^2_1 = 0{\cdot}004$, $P = 0{\cdot}95$). Of the flowering individuals, we were able to collect scent from a total of 18 clonal pairs from all ten Marble Falls seed families and nine clonal pairs from six of the ten Woody seed families planted (Table 1, Table S1). We used these clonal pairs of the same seed family individual as our statistical unit, and thus did not disentangle effects of relatedness at the level of seed family. The reason for this design was that the main target of this study was to compare effects of population affiliation and nutrient treatment on plant trait variation (see Table 1 and Table S1 for more details on sample sizes).

We tested the impact of population origin and nutrient treatment on the number of scapes, the number of leaves, the number of flowers, the scape height and the total floral scent emission rate in multiple linear mixed ANOVA (II) models in the R package nlme. Sample sizes differed slightly between the different response variables, depending on the availability of data from both treatments on each member of the pair (Table 1). Prior to analysis, all data were log-transformed to approach normality and homogeneous variances. In some rare cases, it was possible to obtain data from three or four clones derived from the same bulbil, in which case the values from the high-nutrient treated clones and the low-nutrient treated clones were averaged, respectively. The plant seed family individual (i.e. each clonal pair) was included as a random factor, and plant population (Marble Falls, Woody), nutrient treatment and their interaction were used as categorical (fixed) factors. We tested the effect of nutrient treatment on leaf colour by analysing the average reflectance in each colour spectrum (UV, violet, blue, green, yellow, orange, red) as a repeatedly measured response variable (repeated-measures ANOVA II), with population, treatment and their interaction as factors.

The multivariate variation in floral scent bouquet composition was explored using the vegan package in R. The 19 detected floral scent compounds were used as variables, and a 2-D multidimensional scaling plot based on Bray–Curtis similarities (MDS; 200 restarts) was generated. The similarity of samples of different populations and nutrient levels was tested in a permutational multivariate (perMANOVA) with population and nutrient treatment as factors. Among-population differences in multivariate variance was tested using a permutation test (999 permutations) for homogeneity of multivariate dispersions generated by the function betadisper.

RESULTS

Plants in the low-nutrient treatment produced significantly fewer leaves, scapes and flowers (Table 2, Fig. 1A–C), but the treatment did not affect scape height. Plants of the two populations showed similar variation in these traits in response to nutrient treatment, and the interaction effect of population and nutrient treatment was significant only for number of leaves produced, where only the Marble Falls population showed a reduced leaf set at lower nutrient levels (Table 2, Fig. 1A). Leaves of plants of the low-nutrient treatment were significantly lighter (higher reflectance) than the dark green leaves of the high-nutrient treatment in both populations (Table 1, Fig. 1D).

In contrast, nutrient level had no significant effect on the per-flower floral scent emission (Table 2). Floral scent, however, did vary significantly among populations (Table 2) with Woody plants emitting significantly more scent than the samples from Marble Falls (Fig. 1E). The scent emission rates of the same clone in different treatments were strongly positively correlated ($r^2 = 0{\cdot}73$, $F_{1,25} = 68{\cdot}1$, $P < 0{\cdot}001$), but within populations the correlation was significant only for plants from Marble Falls (Marble Falls, $r^2 = 0{\cdot}61$, $F_{1,16} = 25{\cdot}4$, $P < 0{\cdot}001$; Woody $r^2 = 0{\cdot}23$, $F_{1,7} = 2{\cdot}11$, $P = 0{\cdot}19$) (Fig. 2A).

The floral scent bouquet consisted of a total of 19 compounds. These were mainly aromatics, including several benzenoid alcohols, esters and ethers (Supplementary Data, Table S2). All samples from Woody were dominated by 1,4-DMB, whereas only five of 18 Marble Falls seed family individuals emitted more than trace amounts of 1,4-DMB. In four of these cases, both clones emitted 1,4-DMB, but in one case (8869·1, Table S2) one clone in a pair emitted 1,4-DMB, whereas the other did not, implying either a developmental switch function where the same clonal type can generate different phenotypes (triggered by something other than nutrients), or a technical mishap during plant handling or scent analysis. The nine scent samples that emitted 1,4-DMB clustered closer to the Woody samples in multivariate space than the Marble Falls samples lacking 1,4-DMB (Fig. 2B). The multivariate distributions of the two populations were significantly different (Fig. 2B), but the scent composition was unaffected by nutrient treatment (perMANOVA: population $r^2 = 0{\cdot}42$, $F_{1,50} = 36{\cdot}4$, $P < 0{\cdot}001$; nutrient treatment $r^2 = 0{\cdot}005$, $F_{1,50} = 0{\cdot}45$, $P = 0{\cdot}74$; population

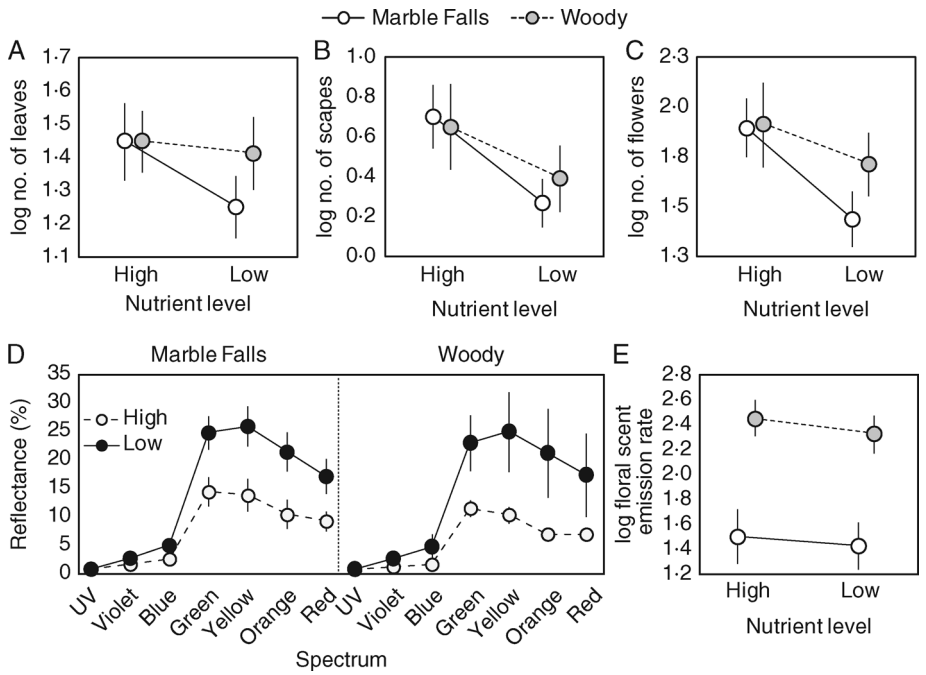

FIG. 1. The effects of population and nutrient treatment on *Lithophragma bolanderi* from Marble Falls (white circles) and Woody (grey circles) in terms of (A) the number of leaves [$n_{\text{Marble Falls}}=32$ (16 clonal seed individual pairs), $n_{\text{Woody}}=16$ (eight pairs)], (B) the number of scapes [$n_{\text{Marble Falls}}=36$ (18 pairs), $n_{\text{Woody}}=22$ (11 pairs)] and (C) the number of flowers produced [$n_{\text{Marble Falls}}=32$ (16 pairs), $n_{\text{Woody}}=9$ (18 pairs)]. Also shown are (D) the reflectance of plants from the two populations grown under different nutrient conditions [white circles = high nutrient; black circles = low nutrients; $n_{\text{Marble Falls}}=34$ (17 pairs), $n_{\text{Woody}}=18$ (nine pairs)] and (E) the effect of population and nutrient treatment on the total standardized floral emission rates [(ng scent per flower) h^{-1}; $n_{\text{Marble Falls}}=36$ (18pairs), $n_{\text{Woody}}=18$ (nine pairs)]. Error bars indicate 95 % confidence intervals around the mean.

TABLE 2. *Statistical output table, reporting the effect of high- and low-nutrient treatment on multiple plant traits of Lithophragma bolanderi tested in linear mixed models (ANOVA II) (a–d, f) or using repeated-measures ANOVA (II) (e)*

	df	*F*	*P*		*df*	*F*	*P*
(a) No. of leaves				(b) No. of scapes			
Population (P)	1	1·06	0·31	Population (P)	1	0·18	0·67
Nutrient Treatment (NT)	1	24·8	**<0·001**	Nutrient Treatment (NT)	1	20·1	**<0·001**
P × NT	1	6·8	**0·016**	P × NT	1	1·06	0·31
Error	22			Error	27		
(c) No. of flowers				(d) Scape height			
Population (P)	1	2·55	0·2	Population (P)	1	0·31	0·58
Nutrient Treatment (NT)	1	21·6	**<0·001**	Nutrient Treatment (NT)	1	2·57	0·12
P × NT	1	2·4	0·14	P × NT	1	1·68	0·21
Error	22			Error	25		
(e) Reflectance				(f) Floral scent			
Population (P)	1	0·99	0·33	Population (P)	1	44·5	**<0·001**
Nutrient Treatment (NT)	1	43·6	**<0·001**	Nutrient Treatment (NT)	1	2·39	0·13
P × NT	1	1·33	0·26	P × NT	1	0·22	0·65
Error	24			Error	25		
Colour Spectrum (CS)	6	688·6	**<0·001**				
CS × P	6	1·11	0·36				
CS × NT	6	7·14	**<0·001**				
CS × P × NT	6	0·19	0·98				
Error	288						

Traits are vegetative (number of leaves; a), reproductive (scape height, number of scapes, number of flowers; b–d), visual (reflectance; e) and chemical (total per-flower volatile emission rate; f) in two *Lithophragma bolanderi* populations (Marble Falls and Woody) in the two nutrient treatments. All response variables were log-transformed prior to analyses. Significant effects are highlighted in bold.

× nutrient treatment $r^2=0{\cdot}008$, $F_{1,50}=0{\cdot}73$, $P=0{\cdot}50$). The presence or absence of 1,4-DMB alone did not explain the entire among-population variation, as populations were significantly different also when this compound was removed from analysis (perMANOVA: population $r^2=0{\cdot}15$, $F_{1,50}=9{\cdot}58$, $P<0{\cdot}001$; nutrient treatment $r^2=0{\cdot}006$, $F_{1,50}=0{\cdot}36$, $P=0{\cdot}91$; population × nutrient treatment $r^2=0{\cdot}031$, $F_{1,50}=1{\cdot}93$, $P=0{\cdot}08$). Benzyl alcohol, dimethyl salicylate and cinnamyl alcohol were all

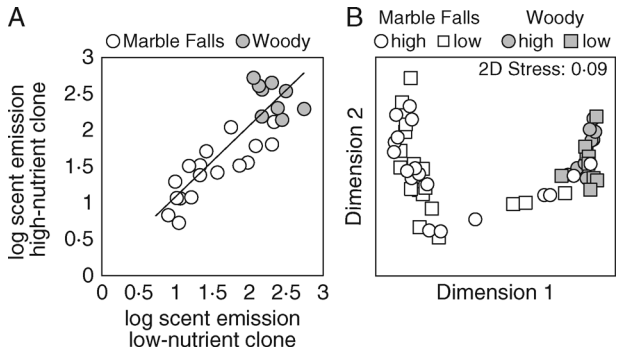

FIG. 2. Floral scent variation in *Lithophragma bolanderi*. In (A) the positive relationship ($r^2 = 0{\cdot}73$) between the total scent production [(ng scent per flower) h^{-1}] in clonal pairs subjected to the low- and the high-nutrient treatment indicates a substantial genetic component on total emission rate. Note, however, that at the within-population level this relationship was significant for Marble Falls (white circles), but not for Woody (dark circles). In (B), the multivariate variation is presented as an MDS plot showing the multivariate distributions of samples from the two populations. Scent variation was larger among samples from Marble Falls (white symbols) than for Woody (dark symbols) (permutation test, $F_{1,52} = 25{\cdot}5$, $P < 0{\cdot}001$), but the high (circles) and low (squares) nutrient treatment did not affect the multivariate variation.

more common in Woody samples, whereas methyl salicylate was stronger in samples from Marble Falls (Table S2).

DISCUSSION

Ecological and evolutionary studies on floral scent have become a major topic in plant research, and several recent studies stress the importance of floral chemistry for fitness and diversification (e.g. Dötterl *et al.*, 2005; Raguso, 2008; Schiestl and Johnson, 2013; Parachnowitsch, 2014; Friberg *et al.*, 2014; Parachnowitsch and Manson, 2015; Suinyuy *et al*, 2015). We tested here a crucial assumption, by disentangling genetic and environmental components for explaining floral scent variation. The overall results suggest substantial canalization in the production of floral scent in *L. bolanderi* under divergent environmental conditions. The same seed family individual grown under different nutrient levels did not differ in floral scent composition or per-flower scent emission rate, but differed strongly in vegetative and reproductive morphological characters. These results imply that floral chemistry just like floral morphology is weakly correlated with vegetative traits (Herrera, 2009; Conner *et al.*, 2014), and is less susceptible to environmental factors than other reproductive or vegetative traits (Mal and Lovett-Doust, 2005; Brock and Weinig, 2007; Burkle and Irwin, 2009; Pélabon *et al.*, 2011). The results held for two populations that differ greatly in the number and composition of floral scents they produce.

Hitherto, not much is known about how costly it is for a plant individual to produce a strong floral scent signal. Previous work implies that the scent signalling could impose both ecological (Kessler and Halitschke, 2009; Theis and Adler, 2012) and energetic costs (Gershenzon, 1994). Many volatiles are produced in pathways that include amino-acid precursors (Weaver and Herrmann, 1997; Pichersky, 2006), which could imply that production costs are disproportionally high under low-nutrient conditions. Evidence from *Abronia umbellata* (Nyctaginaceae) suggests such costs of scent production, because selfing plants that do not need to attract pollinators produce substantially less scent than conspecific obligate outcrossing populations (Doubleday *et al.*, 2013). Also, many plant species, including *L. bolanderi*, tailor their floral scent emission to the time of day when their pollinators are active (Matile and Altenburger, 1988; Raguso *et al.*, 2003; Hoballah *et al.*, 2005; Friberg *et al.*, 2014), or terminate scent emission after pollination (e.g. Shiestl *et al.*, 1997; Negre *et al.*, 2003). Such a shut-down of scent emission outside the period when pollination is likely further implies that unnecessary floral scent signalling is costly for the plant either energetically or ecologically through attraction of enemies. Still, the floral scent of *L. bolanderi* was not compromised even under low-nutrient conditions, whereas plants allocated less energy into leaf material, and flower and scape production.

If scent emission is indeed costly, a largely canalized floral scent signal, like in *L. bolanderi*, could indicate that the floral scent is effectively mediating the interaction with pollinating insect mutualists in each population only when emitted at certain quantity and with particular compound combinations. Previous studies show that *Greya* females preferentially navigate toward the floral scent of their local *Lithophragma* plant species (Friberg *et al.*, 2014, 2016), but the hypothesis that the moth females discriminate also between populations of the same *Lithophragma* species remains to be tested. Furthermore, although the *Greya* moth mutualists are the most common pollinators and the only herbivores that consistently and abundantly attack *L. bolanderi* during egg-laying (Thompson and Pellmyr, 1992; Thompson *et al.*, 2013), the *Lithophragma* plants are sometimes visited also by generalist pollinators such as solitary bees or bombyliid flies (Thompson and Cunningham, 2002; Thompson and Fernandez, 2006). It is possible that geographical variation in the relative importance of the *Greya* specialists and the generalist pollinators could generate floral scent variation among populations.

Most of the few studies that assess phenotypic plasticity in floral scent have focused either on variation between natural conditions and greenhouse common gardens (Majetic *et al.*, 2010; Friberg *et al.*, 2013), or on variation in response to diurnal rhythm or temperature (Matile and Altenburger, 1988; Raguso *et al.*, 2003; Hoballah *et al.*, 2005; Majetic *et al.*, 2009; Friberg *et al.*, 2014). Only one previous study (Majetic *et al.*, in press) has experimentally evaluated the effect of plant nutrient availability on floral scent variation, and very few studies have quantified genetic variation among individuals. Zu *et al.* (2016) established that floral scent was heritable in a focal population of the crucifer *Brassica rapa* (Brassicaceae), which responded significantly to artificial selection. The significantly different, and in multivariate space almost non-overlapping, floral scent composition of the two study populations offers the possibility for using *L. bolanderi* as a model system for future studies aimed at partitioning the heritability of floral scent among and within multiple populations. Furthermore, the strong concordance in floral scent emission between *L. bolanderi* individuals of the same clonal pairs from Marble Falls targets this population for studies that estimate how floral scent variation relates to plant fitness. None of the four studies that have estimated fitness in relation to floral scent in natural populations have identified such links between phenotypic and genetic variation (Schiestl *et al.*, 2011; Parachnowitsch *et al.*, 2012; Ehrlén *et al.*, 2012; Gross *et al.*, 2016).

In summary, this study is one of the first to test the effect of nutrient environment on floral scent emission rate and composition. The results suggest that the among-population variation in floral scent of *L. bolanderi* is largely genetically determined. The largely canalized floral scent emission contrasts starkly with the plastic responses to nutrient treatments by vegetative and other reproductive traits. Hence, our results suggest that some reproductive traits important to coevolving interactions may be more canalized than other traits important for plant fitness.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1. Details on sample sizes. Table S2. Floral scent data, and data on scape length and the number of leaves, scapes and flowers. Table S3. Reflectance data.

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